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## ORT DOCUMENTATION PAGE

1a. <b>AD-A202 689</b>		1b. RESTRICTIVE MARKINGS	
2a.		3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)  NMRI 88-25		7a. NAME OF MONITORING ORGANIZATION Naval Medical Command	
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research	6b. OFFICE SYMBOL (If applicable)	7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, D.C. 20372-5120	
6c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research and Development Command	8b. OFFICE SYMBOL (If applicable)	10. SOURCE OF FUNDING NUMBERS	
8c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055		PROGRAM ELEMENT NO. 62770A	PROJECT NO. 3M16277 OA870
		TASK NO. AF 312-1	WORK UNIT ACCESSION NO. DA301614
11. TITLE (Include Security Classification) Mature Liver Stages of Cloned Plasmodium Falciparum Share Epitopes with Proteins from Sporozoites and Asexual Blood Stages			
12. PERSONAL AUTHOR(S) Szarfman,A; Lyon,J; Walliker,D; Quakyi,I; Howard,RJ;Sun,S; Ballou,WR; Esser,K; London,WT; Wirtz,RA; Carter,R.			
13a. TYPE OF REPORT journal article	13b. TIME COVERED FROM TO	14. DATE OF REPORT (Year, Month, Day) 1988	15. PAGE COUNT 12
16. SUPPLEMENTARY NOTATION reprinted from: Parasite Immunology v.10, no.3, pp.339-351, May 1988			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	Antigens, protozoan; Liver diseases, parasitic; Malaria; Plasmodium falciparum; animal	
19. ABSTRACT (Continue on reverse if necessary and identify by block number)			
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Phyllis Blum, Information Services Division		22b. TELEPHONE (include Area Code) 202-295-2188	22c. OFFICE SYMBOL ISD/ADMIN/NMRI

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## Mature liver stages of cloned *Plasmodium falciparum* share epitopes with proteins from sporozoites and asexual blood stages

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Accepted for publication 7 August 1987

**Summary** The liver merozoites of malaria parasites are of paramount importance, as they initiate the parasite invasion of red blood cells and start the cycle associated with the clinical features of malaria. Investigating liver merozoite antigen is difficult because of the lack of a rodent model of human malaria. In addition, only a low proportion of cells are obtained *in vivo*, the parasites from *Cebus* and *Aotus* monkeys are immature, and *in-vitro* experiments with liver cells are often confounded by contamination with the natural mosquito flora co-purified with the sporozoites used for seeding the liver cultures.

In our study, mature liver schizonts were shown to possess many of the antigenic determinants recognized by MoAbs and sera specific for defined sporozoite and blood-stage antigens. We employed an immunofluorescence procedure based on evaluating parasites in cryosections prepared from infected chimpanzee liver. Sufficient numbers of sectioned parasites were evaluated with each antibody to assure the reproducibility of the results, and the fixation procedure used was sufficiently non-destructive to parasite antigens so that clear differences between reactions of specific antibodies and negative controls were observed. *Key words: epitopes, (KR/KT)* ←

Our evidence for sharing of epitopes by liver merozoites and sporozoites or by liver merozoites and asexual blood-stage parasites raises the possibility that immune responses elicited against sporozoites or asexual stage antigens being considered as vaccine candidates may also act against this important, little-studied stage of the parasite.

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Keywords: *Plasmodium falciparum*, liver merozoites, liver-stage parasites, sporozoites, circumsporozoite protein, blood-stage parasites, antigens, monoclonal antibodies

## Introduction

Malaria infection starts when sporozoites enter the vertebrate host via the bite of an infected mosquito and are transported through the blood to the liver, where they invade hepatocytes and multiply. Each sporozoite generates several thousand merozoites by schizogony (asexual division) in the major amplification system of the malaria life cycle. When mature, these merozoites are released from ruptured liver parenchymal cells and initiate infection of erythrocytes. This process starts the parasite cycle which is associated with the clinical features of malaria. Although much is known about the antigens of sporozoites and blood-stage parasites of *Plasmodium falciparum*, little is known about the antigens of merozoites present in late liver schizonts. Because liver-stage parasites are derived by differentiation and amplification of sporozoites, the two stages may share antigens, and studies of liver parasites could therefore be important for development of a sporozoite vaccine. Because both liver merozoites and blood-stage merozoites invade erythrocytes, antigenic studies of liver merozoites could identify shared antigens. This knowledge would also be important for vaccine studies directed against merozoites of either stage. Investigating liver merozoite antigens is difficult because of the lack of a laboratory rodent model of human malaria. In addition, only a low proportion of infected cells are obtained *in vivo*, the parasites from *Aotus* and *Cebus* monkeys are immature (Sodeman *et al.*, 1969, Druilhe *et al.* 1982), and *in-vitro* experiments with liver cells are inherently difficult. Human hepatocyte cultures are frequently contaminated with the natural mosquito flora co-purified with the sporozoites used for seeding (Zavala *et al.* 1985, Mazier *et al.* 1986a); the parasites produced are smaller than those obtained *in vivo* in humans and chimpanzees (Shortt *et al.* 1951, Bray 1958, Bray & Gunders 1962); and mature schizonts are obtained inconsistently (Mazier *et al.* 1985, 1986b), making the evaluation of parasite properties under such conditions very difficult.

Previous studies of non-human malaria indicated that the more mature liver-stage parasites developed *in vivo* may share as yet undefined antigens with blood-stage parasites (Danforth, Orjih & Nussenzweig 1978). Only traces of cross-reactivity between mature liver-stage parasites and sporozoites were present (Danforth *et al.* 1978, Hollingdale *et al.* 1981). Similar results were reported when one monoclonal antibody (MoAb) to sporozoites was used to follow the development of *P. vivax* liver stages *in vitro* (Hollingdale *et al.* 1985). When the liver of a *Cebus apella* monkey (a primate that does not develop blood infection (Druilhe *et al.* 1984)) infected with incompletely developed *P. falciparum* liver stages was examined, no reactivity was observed with MoAbs to sporozoites or blood stages, in contrast to previous findings (Danforth *et al.* 1978). Technical problems, arising from using parasites that may not have developed sufficiently to begin expressing blood-stage antigens or that may have lost immunoreactivity due to the fixation and processing protocols, could have contributed to these conflicting data.

In our study, mature liver schizonts were shown to possess many of the antigenic determinants recognized by MoAbs and sera specific for defined sporozoite and blood-stage antigens. We employed an immunofluorescence procedure based on evaluating parasites in cryosections prepared from infected chimpanzee liver. Sufficient numbers of

sectioned parasites were evaluated with each antibody to assure the reproducibility of the results, and the fixation procedure used was sufficiently non-destructive to parasite antigens so that clear differences between reactions of specific antibodies and negative controls were observed.

## Materials and methods

### CLONE OF *P. FALCIPARUM* PARASITES

Clone HB3 was derived by Dr W. Trager by microscopic selection of cultured blood stages from the Honduras I/CDC strain (Bhasin & Trager 1984).

### SPOROZOITE PRODUCTION AND ISOLATION

A group of approximately 1500 *Anopheles freeborni* mosquitoes were fed by a membrane-feeding apparatus on cultured mature gametocytes of *P. falciparum* (Ifediba & Vanderberg 1981) of clone HB3. After 1 week 10–300 oocysts (average 37) were found in the midgut wall, and after 16 days 5000–20 000 sporozoites (average 10 000) were found in the salivary glands of the mosquitoes. To collect sporozoites the infected mosquitoes were processed as described (Ozaki, Gwadz & Godson 1984) except that heads and abdomens were discarded and the thoraxes were washed six times by centrifugation with Grace's insect medium (GIBCO) containing 10% human AB serum to increase the number of sporozoites collected. The sporozoites were then resuspended in the same medium.

### MATURE LIVER STAGES OF *P. FALCIPARUM*

To obtain late liver schizonts, a chimpanzee received sporozoites from clone HB3. The chimpanzee was infected in two ways as follows: (a) from the bite of infected mosquitoes and (b) from sporozoites isolated from mosquitoes and injected intravenously. The infected mosquitoes were allowed to feed for 15 min on a splenectomized chimpanzee lightly anaesthetized with ketamine, followed 2 h later by intravenous inoculation with  $2 \times 10^6$  purified sporozoites. Six days after infection, a liver wedge biopsy was obtained while the animal was under ketamine and methoxyflurane anaesthesia. Pieces of tissue approximately 1 cm<sup>3</sup> were embedded in Tissue-Tek (Miles Scientific), wrapped in aluminium foil, frozen in ethanol/dry ice and stored at  $-70^\circ\text{C}$ . An average of one liver schizont per 0.5 cm<sup>2</sup> section was found by microscopic examination, and the chimpanzee developed positive blood parasitaemia.

### IMMUNOFLUORESCENCE ASSAYS (IFA) WITH LIVER SCHIZONTS

Tests to study the antigenic composition of liver schizonts were performed double-blind on coded specimens prepared from 2- $\mu\text{m}$  cryostat sections of infected chimpanzee liver. Because the chimpanzee had previously been used in studies on hepatitis viruses, residual viruses were killed by fixing the cryostat sections of infected chimpanzee liver with 1% formalin in Dulbecco's phosphate-buffered saline (PBS) for 10 min. The fixed sections were washed three times for 10 min with PBS, dried, wrapped in aluminium foil, and stored at  $-70^\circ\text{C}$  for not more than 4 weeks until use. Due to the scarcity of parasites in the

liver, every fifth cryostat section was stained with Giemsa, scanned by light microscopy, and sections between parasite-containing sections (which remained frozen until used) were selected as antigens for the immunofluorescence procedure. The liver sections were examined for reactivity with a panel of MoAbs or polyclonal antisera against defined antigens from sporozoites and blood stages. Sections were incubated with antibody for 30 min, washed three times with PBS, incubated with the appropriate fluorescein-labelled second antibody for 30 min and washed again. At least 20 liver-stage schizonts were examined for each reaction with MoAbs or polyclonal mouse or rabbit antisera. An appropriate positive control (a MoAb or a rabbit or mouse serum) and pools of appropriate negative controls (pools of 20–50 MoAbs of different isotypes against *Trypanosoma brucei rhodesiense* and *Rickettsia typhi* (Hall & Esser 1984, Dasch 1981) or pools of 20–50 mouse or rabbit sera) and a PBS control were interjected at random between each set of 10 antibody samples examined. All MoAbs were used at a concentration of 10–40 µg/ml, and all rabbit or mouse sera were used at 1:50 dilution. Fluorescein-labelled goat anti-mouse immunoglobulin (IgA, IgG and IgM) and fluorescein-labelled goat anti-rabbit IgG were obtained from Cooper Biomedical, Malvern, PA, USA, and used at a concentration that precluded false positive reactions. Liver schizonts were identified by phase contrast and fluorescent microscopy, located by vernier coordinates, photographed with Kodak Ektachrome 400 film, then stained with Giemsa and located again for additional photographs. It took an average of 20 min to find each liver schizont.

#### IFA WITH SPOROZOITES AND ASEXUAL AND SEXUAL BLOOD STAGES

The reactivity of monoclonal and polyclonal antibodies against defined antigens was tested with sporozoites and asexual and sexual blood stages of the HB3 clone. Slides containing sporozoites and asexual and sexual blood stages were prepared by spreading 10, 10, or 2 µl, respectively, of a suspension of parasites on to each well of multiwell printed IFA slides. Sporozoites (see above) resuspended in Medium 199 with 2.5% bovine serum albumin were used at a concentration of  $2-5 \times 10^4$ /ml. Asexual blood stages and gametocytes (Lambros & Vanderberg 1979, Ifediba & Vanderberg 1981) were suspended at  $1-2 \times 10^5$ /ml in PBS. The slides were air dried at room temperature, fixed in 1% formalin as previously mentioned and stored at  $-70^\circ\text{C}$  until tested in an IFA.

#### MONOCLONAL ANTIBODIES AGAINST SPOROZOITES

The MoAbs against sporozoites used in these experiments—MoAb 2F1.1, 4D11.6, 5H12.7, 4F2.5, 4D9.1, 1G3.4 and 5G5.3 (Table 1)—were previously described (Hockmeyer & Dame 1985). Further characterization was performed using the ELISA technique with R32tet32 (Young *et al.* 1985), which contains sequences from the repeat domain of the circumsporozoite (CS) protein, or (NANP)<sub>4</sub> (a 16 aa synthetic peptide antigen consisting of four repeats of the *P. falciparum* CS protein) as antigens; and by studying the ability of MoAb 2F1.1 (against sporozoites) to inhibit the binding of those MoAbs to CS protein expressed in *Escherichia coli* (Dame *et al.* 1984). The ELISA was performed as described (Dame *et al.* 1984) except that blocking experiments were done with MoAbs diluted in 0.05% PBS-Tween incubated with antigen for 1 h and washed prior to the addition of peroxidase-conjugated MoAbs. All MoAbs to sporozoites reacted with R32tet32, and all but MoAb 1G3.4 reacted with (NANP)<sub>4</sub>. The binding of all

**Table 1.** Reactivity of antibodies against sporozoites of *P. falciparum* with late liver-stage schizonts

MoAb or antiserum	Ig class	Immunofluorescence reactivity*		
		Sporozoites	Asexual blood stages	Liver schizonts
MoAb 2F1.1	M	++++	—	++
MoAb 4D11.6	M	++++	—	+
MoAb 5H12.7	M	++++	—	+
MoAb 4F2.5	M	++++	—	+
MoAb 4D9.1	G1	++++	—	+
MoAb 1G3.4	G1	++++	—	+
MoAb 5G5.3	G1	++++	—	—
†MA R32tet32	Polyclonal	++++	—	++

\* Fluorescence was graded from — to + + + +.

† MA, Mouse antiserum.

MoAbs except 1G3.4 and 5G5.3 to CS protein could be inhibited by MoAb 2F1.1, suggesting that these MoAbs recognize at least three distinct CS epitopes. A polyclonal mouse serum raised against R32tet32 was also tested by immunofluorescence for reactivity with late liver schizonts. The blocking reaction with R32tet32 was performed at an antigen concentration of 30  $\mu\text{g/ml}$  for 1 h at 37°C and overnight at 4°C, before testing the fluorescence reactivity of the antibodies with liver merozoites. These antibodies reacted with sporozoites but not with asexual blood-stage parasites of the HB3 clone.

#### MONOCLONAL ANTIBODIES AGAINST ASEXUAL BLOOD-STAGE PARASITES

The following antibodies were used, and they identified different epitopes of antigens of blood-stage parasites (Table 2): (i) MoAbs Pf 12-8B7.4 and Pf 9-4H9.1 recognize two distinct epitopes present in Pf EMP2, a 300 kD antigen located on the cytoplasmic face of the erythrocytic membrane of infected red blood cells and within developing parasites (Howard *et al.* 1987); (ii) a rabbit antiserum prepared against affinity-purified Pf EMP2 (Howard *et al.* 1987); (iii) MoAbs raised against the polymorphic 195 kD glycoprotein (gp 195), which is a precursor to a number of antigens on the surface of blood-stage merozoites (Holder & Freeman 1984), recognize different epitopes of gp195 (Lyon *et al.* 1987), MoAb Pf 12-7B2 recognizes the 83 kD proteolytic fragment of gp195, Pf 2-7H10 recognizes the two 45 kD proteolytic fragments of gp195, one of them glycosylated, the other non-glycosylated, and Pf 6-3B10, Pf 12-7F1 and Pf 4-4G12 recognize different epitopes of the glycosylated 45 kD proteolytic fragment of gp195; (iv) MoAb Pf 12-5E3 recognizes an epitope present in a 113 kD antigen and MoAb Pf 12-3D5 recognizes an epitope present in a 101 kD antigen, both of which localized by IFA to the blood-stage merozoite surface as well as the parasitophorous vacuole (Chulay *et al.* 1987); (v) MoAbs Pf 12-4D99, Pf 2-1A2, and Pf 2-1A7 recognize an epitope present in a series of antigens of M, 80, 60, and 40 kD that corresponds to one of the sets of antigens associated with the rhoptry organelles (Campbell *et al.* 1984); (vi) MoAb 12.3 recognizes a clone-restricted

**Table 2.** Reactivity of antibodies against blood stages of *P. falciparum* with late liver-stage schizonts

MoAb or antiserum	Ig class	M <sub>r</sub> of proteins of blood stages recognized by antibodies (kD)	Immunofluorescence reactivity*	
			Blood stages	Liver schizonts
Antibodies against proteins of asexual blood stages				
MoAb Pf 12-8B7.4†	G1	300	÷ + + +	+
MoAb Pf 9-4H9.1†	M	300	++	+
RA† Pf EMP2	Polyclonal	300	++	—
MoAb Pf 12-7B2†	G1	195	++++	++++
MoAb Pf 2-7H10†	G2a	195	++++	++++
MoAb Pf 6-3B10†	M	195	++++	++++
MoAb Pf 12-7F1†	G	195	++	++
MoAb Pf 4-4G12†	G	195	++	++
MoAb Pf 12-5E3	G2a	113	+	+
MoAb Pf 12-3D5	G1	101	++++	++
MoAb Pf 2-4D9	G2a	80, 60, 40	++++	++
MoAb Pf 2-1A2	G2a	80, 60, 40	+++	++
MoAb Pf 2-1A7	G2a	80, 60, 40	++++	++
MoAb 12.3	G!	40	—§	—
MoAb Pf 11-6B3†	G2a	14	++++	++
MoAb Pf 11-2A6†	G1	14	++	+
Antibodies against histidine-rich proteins of asexual blood stages				
MoAb 89 Pf HRP1	G2a	120–80	++++	—
RA MCI Pf HRP1 & 2	Polyclonal	120–180, 85–60	++++	—
RA 6211 P1 HRP	Polyclonal	120–80, 85–60	++++	—
MoAb 87 Pf HRP2	M	85–60	++++	—
RA 4059/60 Pf HRP2	Polyclonal	85–60	++++	—
RA 4123/3 Pf HRP3	Polyclonal	21	—§	—
Antibodies against sexual blood stages				
MoAb IIC5-B10†	G2a	48, 45	++++	—
MoAb IA3-B8†	G2a	48, 45	++++	—
MoAb 3E12†	G1	48, 45	++++	—

\* Fluorescence was graded from — to + + + +, parasites of the HB3 clone were used as antigen.

† Each of those MoAbs recognizes a different epitope (R. J. Howard & J. A. Lyon, unpublished observations, Lyon *et al.* 1987, A.J. Saul & R.J. Howard, unpublished observations, R. Carter, unpublished observations).

‡ RA, Rabbit antiserum.

§ + + + + with asexual blood stages of clone 3D7.

epitope present in a 40 kD protein that is associated with the surface of schizonts and merozoites (McBride, Welsby & Walliker 1984, J.S. McBride & D. Walliker, unpublished observations); and (vii) MoAbs Pf 11-6B3 and Pf 11-2A6 recognize two different epitopes of a 14 kD protein that appears to be associated with the merozoite surface (J.A. Lyon, unpublished observations). All these antibodies except MoAb 12.3 reacted with the

asexual blood stages of the HB3 clone. MoAb 12.3, which did not react with asexual blood-stage parasites of the HB3 clone, reacted strongly with asexual-stage parasites of clone 3D7 derived by limiting dilution (Rosario 1981) from isolate NF54 from the Netherlands (Ponnudurai, Leeuwenberg & Meuwissen 1981).

Some of the antibodies tested identify different *P. falciparum* histidine-rich proteins (Pf HRP1, Pf HRP2, or Pf HRP3) of asexual blood stages (Wellems *et al.* 1987): MoAb 89 recognizes a specific epitope of Pf HRP1: a 80–120 kD protein located under the knobs at the infected erythrocyte membrane and in organelles in the host cell cytoplasm (D.W. Taylor, M. Aikawa & R.J. Howard, unpublished observations); MoAb 87 recognizes a specific epitope of Pf HRP2 (Wellems & Howard 1986), a 85–60 kD protein secreted from intact infected erythrocytes and also found in the cytoplasm of host cell and parasite (Howard *et al.* 1986); rabbit serum MC1, raised against Pf HRP1, recognizes different epitopes present in Pf HRP1 and Pf HRP2; rabbit sera 4059 and 4060, raised against the synthetic peptide Ala-His-His (Ala-His-His-Ala-Ala-Asp)<sub>2</sub> of Pf HRP2 and specific for this HRP; rabbit sera 4132 and 4133 raised against the pentapeptide repeat Asp-Gly-Ala-His-His of Pf HRP3, and specific for this HRP, of calculated molecular weight 21 kD; and rabbit serum 6211 raised against *P. lophurae* HRP, a 45–50 kD protein, recognizes different epitopes present in Pf HRP1 and Pf HRP2 (Rock *et al.* 1987). All of the MoAbs and rabbit antisera which reacted with Pf HRP1 and/or Pf HRP2 reacted with asexual blood-stage parasites of clone HB3, whereas the rabbit antisera specific for Pf HRP3 reacted with asexual blood-stage parasites from clone 3D7 but not with HB3 clone.

#### MONOCLONAL ANTIBODIES AGAINST SEXUAL BLOOD-STAGE PARASITES

Several MoAbs (IIC5-B10, IgG2a, IA3-B8, IG2a and 3E12, IgG1) specific for the sexual blood gametocytes of *P. falciparum* were also tested (Table 2). Each one recognized different epitopes of gametocyte proteins when tested by two-site immunosay. These MoAbs reacted by immunofluorescence with gametocytes of the HB3 clone. When they were tested by immunoblotting of antigens from gels run under non-reducing conditions they reacted with gametocyte proteins of M<sub>r</sub> 48 and 45 kD (Rener *et al.* 1983, R. Carter, unpublished observations).

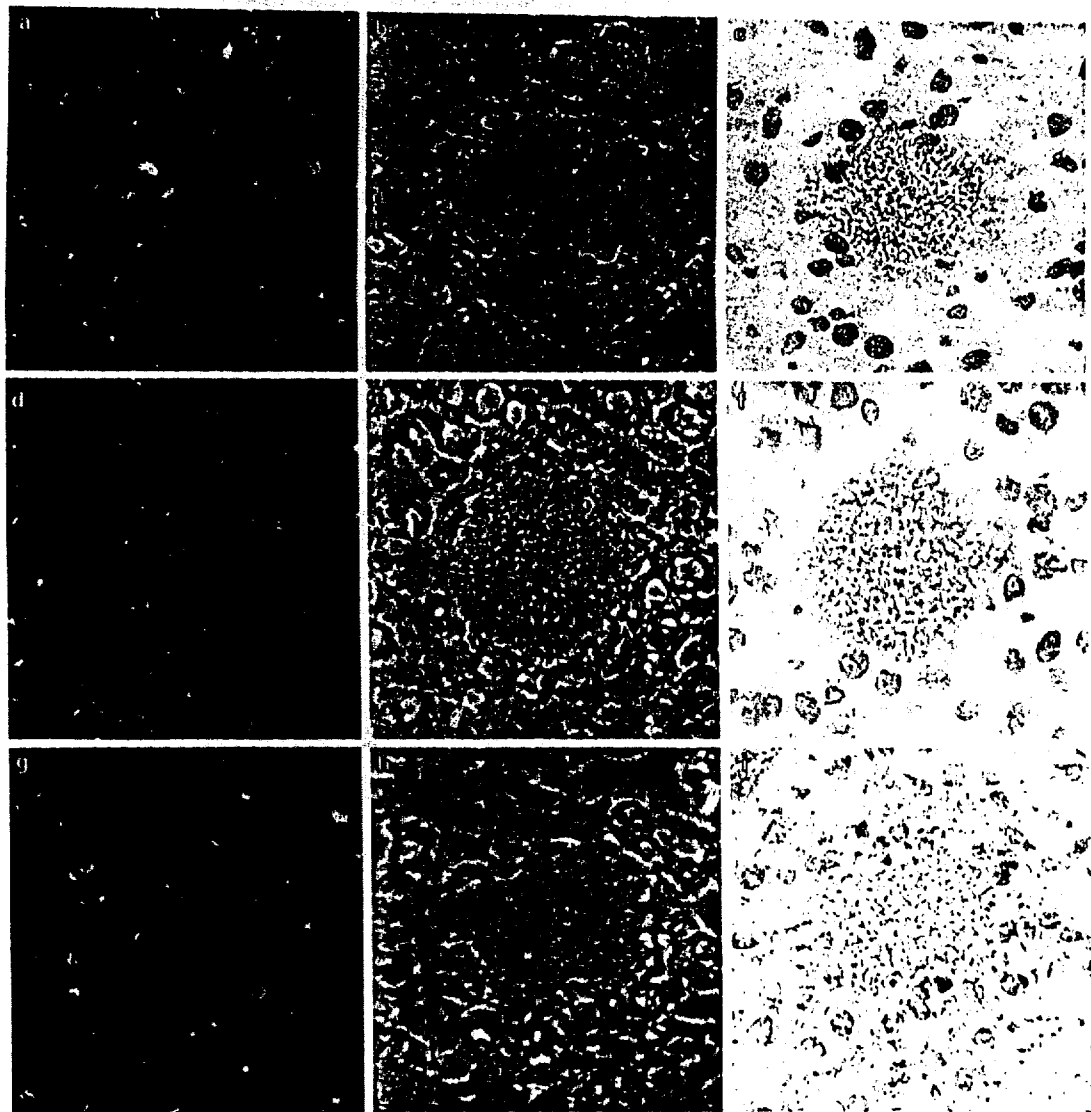
### Results

#### REACTIVITY WITH ANTIBODIES TO SPOROZOITES

As seen in Table 1, six of seven of the MoAbs against sporozoites and mouse monospecific antibodies to R32tet32 serum reacted with late liver schizonts, and showed a fine granular IFA pattern (Figure 1a). Furthermore, this reaction could be inhibited specifically by R32tet32. The fluorescent reaction of these antibodies with late liver schizonts was not as intense as with intact sporozoites. None of these antibodies reacted with blood-stage parasites.

#### REACTIVITY WITH ANTIBODIES TO ASEXUAL BLOOD-STAGE PARASITES

As seen in Table 2, mature liver schizonts possess many, but not all, of the antigenic specificities of asexual blood stages. In general, the MoAbs that recognize late liver



**Figure 1.** Top row: (a) Indirect immunofluorescence of a liver schizont of *P. falciparum* showing a fine granular pattern, graded as +; (b) phase microscopy, and (c) Giemsa staining of the same parasite. Middle row: (d) Indirect immunofluorescence of a liver schizont of *P. falciparum* showing a coarse granular pattern, graded as +++; (e) phase contrast microscopy, and (f) Giemsa staining of the same parasite. Bottom row: (g) Indirect immunofluorescence of a liver schizont of *P. falciparum* showing a negative pattern; (h) phase microscopy, and (i) Giemsa staining (phase contrast) of the same parasite. Original magnification  $\times 312.5$ .

schizonts reacted less strongly with liver schizonts than with the blood-stage parasites. An exception to this observation was the MoAbs to gp195 which reacted with equal fluorescence intensity when liver schizonts and blood-stage parasites were tested. MoAbs to the 300 kD and the 80, 60, and 40 kD proteins gave a fine granular pattern, while the rest of the MoAbs which reacted with late liver schizonts produced a coarse granular pattern (Figure 1d). The following specific antibodies against asexual blood-stage antigens did not react with late liver schizonts of the HB3 clone: the rabbit antiserum against PfEMP2; MoAb 12.3, which defines an epitope against a 40 kD protein; and all of the MoAbs and rabbit sera which reacted with Pf HRP1, Pf HRP2 and Pf HRP3.

#### REACTIVITY WITH ANTIBODIES TO SEXUAL BLOOD-STAGE PARASITES

MoAbs that define different epitopes of gametocyte proteins did not react with late liver schizonts of the HB3 clone (Figure 1g).

#### Discussion

The significance of this study lies in the demonstration that late liver schizonts which are distinct from other stages of the parasite, share some defined antigenic determinants with sporozoites and asexual-stage parasites.

Our study shows that antibodies against at least two epitopes of the CS protein of sporozoites and 13 epitopes on at least seven proteins from asexual blood-stage parasites also reacted with late liver-stage schizonts. Antibodies against other antigens of asexual blood stages and gametocytes did not react with liver schizonts. Liver merozoites are the parasites that will first invade red blood cells, and must synthesize proteins with critical functions in erythrocyte recognition, attachment, and entry. These proteins and corresponding epitopes are probably similar, if not identical, to the analogous blood-stage merozoite protein. Conversely, antigens that are not expressed by both liver-stage merozoites and blood-stage merozoites probably do not contribute to invasion. This could be the case for epitopes of the histidine-rich proteins whose functions are not known, or epitopes of gametocyte proteins that presumably have functions associated with gametogenesis, fertilization and zygote development in the mosquito midgut and are not needed in liver merozoites.

The weaker reactions of some MoAbs with late liver schizonts could be due to a lesser amount of antigen present in liver schizonts, to the masking of some antigenic determinants due to their interaction with other components of the liver-stage parasites, or to a lower affinity of the interaction. In addition, we cannot rule out the possibility that the MoAbs cross-react with liver-stage antigens that are unrelated functionally to blood-stage antigens. The reactivity of late liver schizonts with anti-sporozoite antibodies observed here is probably not due to the cross-reacting proteins of asexual blood stages (Hope *et al.* 1984, Coppel *et al.* 1985), because the antibodies to the CS protein of sporozoites tested did not react with infected red blood cells. Although proteins present in different developmental stages may share epitopes, the proteins may not be identical. This concept is supported by our results with antibodies raised against the  $M_r$  300 kD (Pf EMP2) protein of asexual *P. falciparum* parasites. Monoclonal antibodies 8B7.4 and 4H9.1 reacted with both asexual and liver-stage parasites, whereas rabbit antiserum raised against the Pf EMP2 antigen (which reacts with the same protein as the MoAbs in asexual stage parasites) reacted only with asexual parasites.

Since a single sporozoite escaping immune surveillance could produce several thousand liver merozoites and induce clinical malaria, it is possible that sporozoite vaccines alone will not be sufficient to induce effective protection, and that antigens of asexual blood stages will be needed to provide immunity against these stages as well. Our results suggest that due to shared epitopes, immune responses against sporozoites or asexual blood-stage parasites induced after natural infection or immunization could also act upon liver-stage merozoites and thereby reduce the first parasite load upon erythrocytes. Conversely, immune mechanisms induced by liver merozoites that have a

total mass several thousand times larger than sporozoites could act upon sporozoites and/or asexual blood stages and reduce the parasite load upon liver cells and/or erythrocytes. The lack of inflammatory-cell-infiltrates around intact liver schizonts as observed previously (Garnham 1966) and confirmed in the present study could possibly indicate a lack of expression or recognition of parasite antigens on the surface of infected liver cells, suggesting that immune responses to those common antigens only start to act in the non-immune animal after the liver merozoite is released. The possibility that immune recognition in immune animals could be initiated earlier, at the developing liver stage, should be further investigated.

An understanding of the antigenic composition of liver merozoites, due to the pivotal role that these parasites play between the asymptomatic liver stage and blood stage associated with clinical disease, could have important implications for malaria vaccine development.

### Acknowledgements

This work was supported by Naval Medical Research and Development Command work unit 3M162770A870AF312. We thank R. Wistar Jr, L.H. Miller, F.A. Neva, W.T. Hockmeyer and W.E. Collins for valuable advice and discussion. We are grateful to T. Ponnudurai for providing the NF54 isolate of *P. falciparum*; to P. Nguyen-Dinh for the clone HB3 of *P. falciparum*; to A.J. Saul for the rabbit antiserum to Pf EMP2; to D.W. Taylor and J. Rener for MoAbs 87 and 89; to T.E. Wellems and W.L. Maloy for the rabbit antiserum to Pf HR23; to G. Dasch for MoAbs to *Rickettsia typhi*; to S. Naum and G. Cotten for technical assistance; and to B.J. Leckey for editorial assistance. I. Quakyi is a recipient of financial support from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

### Notes

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the US Navy or the naval service at large.

The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services Publication No. (NIH) 85-23.

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